

***In vitro* effects and mechanisms of lycopene in MCF-7 human breast cancer cells**

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ABSTRACT. Breast cancer adversely affects the health status of women; therefore, the prevention and treatment of breast cancer is of critical importance. Lycopene is known to possess several biological effects such as removal of free radicals, alleviation of biological oxidative injury, and inhibition of tumor growth. In this study, we aimed to illustrate the effect of lycopene on tumor cell proliferation and modulation of cancer progression as well as its possible underlying mechanisms in human breast carcinoma cell line MCF-7 *in vitro*. MCF-7 cells were treated with different lycopene concentrations for 24, 48, and 72 h. Light field microscopy was used to observe cell morphology. MTT assay was used to determine the effect of lycopene on MCF-7 proliferation. Flow cytometry was employed to evaluate cell apoptosis. Real-time quantitative polymerase chain reaction was performed to detect the expression of p53 and Bax. Under microscopic examination, the untreated MCF-7 cells appeared to have a diamond or polygonal shape. Lycopene treatment resulted in cell shrinkage and breakage, whose severity increased in a dose and duration dependent manner. In addition, reduced cell proliferation and increased apoptosis ($P < 0.05$)

were observed using MTT assay and flow cytometry, respectively. Moreover, lycopene could also upregulate the expression of p53 and Bax mRNAs in MCF-7 cells. In conclusion, lycopene inhibits proliferation and facilitates apoptosis of MCF-7 cells *in vitro*, possibly by regulating the expression of p53 and Bax.

Key words: Lycopene; MCF-7 cells; p53; Bax; Breast cancer;
In vitro study

INTRODUCTION

The incidence of breast cancer has increased globally, and it has become the most common malignant cancer in women. Breast cancer severely affects women health. The development of modern medicine has largely improved the early diagnosis rate of cancer, and it has resulted in drugs and treatment approaches that can lead to increased survival rate in patients. Based on the pathological types, stages, and the biological activities of patients, various treatment strategies such as surgery, radiation, endocrine therapy, chemotherapy, molecular target therapy, Chinese medicine, and immunotherapy can be selected. However, all these methods have certain adverse effects and variable efficacy. Therefore, the establishment of anti-tumor drugs with specific gene locus, wide spectrum, high efficacy, and low toxicity is of critical importance for the treatment of breast cancer.

Lycopene is a carotenoid pigment found in red vegetables and fruits, especially in tomatoes. It did not draw much attention previously because it lacks vitamin A activity. However, recent studies have revealed the potential roles of lycopene, such as removal of free radicals, alleviation of bio-oxidative stress, modulation of body immune functions, and prevention of cardiovascular disease (Trejo-Solis et al., 2013; Cefali et al., 2015; Ilahy et al., 2016). In addition, lycopene can exert anti-tumor effects via regulation of cell growth factor signaling pathways, initiation of cell cycle arrest, and induction of cell apoptosis (Ono et al., 2015). Being a natural component in daily diet, lycopene has several advantages such as fewer adverse effects and easy extraction method, which attract extensive research interests in its anti-tumor effects. Previous findings showed that the intake of pro-lycopene was closely correlated with the incidence of prostate cancer (Wang et al., 2015a,b). Lycopene concentration in patients with pulmonary cancer was significantly lower than that in healthy individuals (Asbaghi et al., 2015). However, the role of lycopene in breast cancer is still controversial. One study showed that carotenoid intake was negatively correlated with estrogen receptor [ER (-)] breast cancer, but not with ER (+) cases (Zhang et al., 2012). Another study suggested that the intake of peripheral carotenoids, including lycopene and β -carotid, could decrease the risk of breast cancer, especially for the ER (-) subtypes (Eliassen et al., 2012).

The tumor suppressor gene p53 plays an important role in the regulation of cell cycle, as it arrests cell growth from G phase into S phase, thereby inducing cell apoptosis (Sohn et al., 2003). Overexpression of the pro-apoptotic gene Bax can induce cell apoptosis (Rampino et al., 1997). Bax is also a downstream effector of early p53 response gene (Brady and Gil-Gómez, 1998). A previous study showed cell cycle arrest by lycopene (Takeshima et al., 2014). Given the contradicting results obtained from previous studies, the exact role of lycopene in MCF-7 cells, which is an ER (+) breast cancer cell line, remains poorly understood. In this study, we aimed to investigate the effect of lycopene in MCF-7 cells by treating them with

different concentrations of lycopene. The mRNA levels of p53 and Bax gene were determined to explore the possible effects and related mechanisms of lycopene in MCF-7 cells.

MATERIAL AND METHODS

Reagents

Human breast carcinoma cell line MCF-7 was purchased from ATCC cell bank (USA). The reagents used in this study are: RPMI 1640 culture medium (Gibco, Carlsbad, CA, USA); fetal bovine serum (FBS; Hyclone, Logan, UT, USA); streptomycin-penicillin dual antibiotic solution (Gibco); Dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), and apoptotic test kits (Beyotime Biotechnology, Shanghai, China); Trizol RNA extraction kit (Invitrogen, Carlsbad, US); real-time fluorescent polymerase chain reaction (qPCR) test kit (TaKaRa Biomedicals, Kusatsu, Shiga, Japan); gel imaging system and fluorescent PCR cyler model ViiA7 (ABI, Thermo Fisher Scientific, Waltham, MA, USA).

Cell culture

Human breast cancer MCF-7 cells were cultured in RPMI 1640 medium containing 10% FBS, 100 U/mL streptomycin, and 100 U/mL penicillin in a humidified chamber with 5% CO₂ at 37°C. The cells were passaged every 2-3 days. During passaging, the culture medium was discarded, followed by rinsing twice with PBS. Then, the cells were digested by treatment with 0.25% trypsin for 30 s to harvest adherent cells for the preparation of single-cell suspensions, which were centrifuged at 1000 g for 5 min. The supernatants were discarded, and the cells were re-suspended for cell culture.

MTT assay

The cells at log phase were inoculated on 96-well plates, with 5 x 10³ cells in each well. A complete medium of RPMI 1640 was added to incubate cells for 24 h, at 37°C under 5% CO₂. The cells were treated with different concentrations of lycopene (0, 2, 4, 8, and 16 μM) for different durations (24, 48, and 72 h) and with the blank control, which contained only 200 μL culture medium, in parallel. Lycopene treatment was performed in five parallel wells for each concentration. After treatment, 20 μL MTT working solution (5.0 g/L) was added to each well and incubated for 4 h. The supernatants were then discarded. MTT-formazon crystals were dissolved by the addition of 150 μL DMSO for 10 min until complete dissolution of the purple crystals. The absorbance (A) value in each well at 490 nm was measured.

$$\text{Cell survival rate} = (A_{\text{Experimental group}} - A_{\text{Blank}}) / (A_{\text{Control}} - A_{\text{Blank}})$$

Flow cytometry

The cell suspensions were added on a 6-well plate, which was acclimated for 24 h. Then, different concentrations of lycopene (0, 2, 4, 8, and 16 μM) were added, followed by subsequent incubation for different durations (24, 48, and 72 h) in triplicates. After lycopene treatment, cell morphology was observed using light field microscopy. Trypsin was used to digest the cells. The cells were then rinsed twice with PBS and centrifuged at 1000 g for 5 min. The cells (1 x 10⁶) were

re-suspended in PBS and centrifuged at 1000 *g* for 5 min. The supernatants were discarded, and the cells were re-suspended in 195 μ L binding buffer, followed by the addition of 5 μ L Annexin V-FITC. Then, the cells were incubated in propidium iodide (10 μ L, PI) solution for 20 min at 25°C in the dark. The apoptosis rate was measured using flow cytometry.

mRNA assay

Total RNA was extracted with Trizol reagent. The purity and concentration of RNA were determined by Nano Drop (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was evaluated by 1% agarose gel electrophoresis. cDNA was synthesized from RNA (1 μ g) using a reverse transcription kit (TaKaRa). Quantitative PCR was performed by using real-time fluorescent test kit. The reaction mixture comprised 5 μ L 2X SYBR Green Mixture, 0.5 μ L cDNA, 0.5 μ L primer (10 μ M), and 4.0 μ L ddH₂O under the following conditions: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and annealing at 60°C for 60 s on a model ViiA7 fluorescent quantitative PCR cyclers in triplicates, using β -actin as the internal reference.

Statistical analysis

SPSS19.0 software was used to perform statistical analysis. Data following a normal distribution are reported as means \pm standard deviation (SD). Analysis of variance (ANOVA) was used for analysis of data. Fisher's Least Significant Difference (LSD) test was used to compare two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of lycopene on MCF-7 cell morphology

After lycopene treatment at different concentrations, the morphology of MCF-7 cells was observed under an inverted microscope. Untreated MCF-7 cells appeared to have a diamond or polygonal shape with normal proliferation (Figure 1). The number of MCF-7 cells decreased after lycopene treatment, and they showed shrinkage and breakage. These phenomena were aggravated with increasing concentrations and treatment durations.

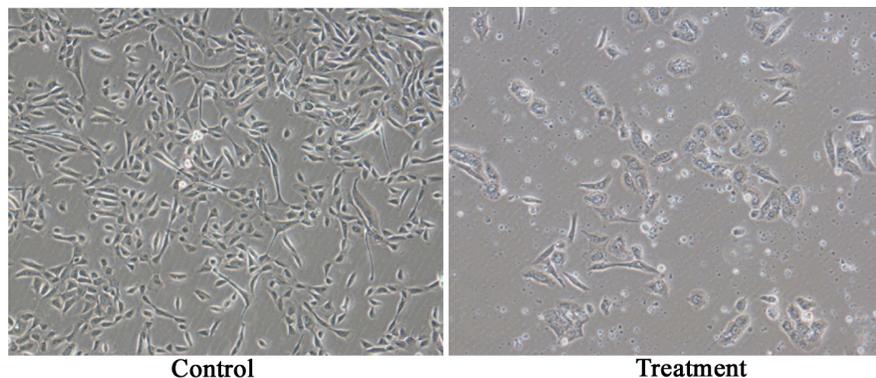


Figure 1. Effects of lycopene on MCF-7 cell morphology.

Effects of lycopene on MCF-7 cell proliferation

MTT assay was used to test the viability of MCF-7 cells treated with different lycopene concentrations for 24, 48, and 72 h. Lycopene significantly inhibited MCF-7 cell proliferation in a dose- and time-dependent manner. Significant inhibitory effects were observed with increasing concentrations or longer treatment periods (Table 1). In particular, the most potent inhibitory effects on cell proliferation were observed in cells treated with 16 μ M lycopene for 72 h.

Table 1. Effects of lycopene on MCF-7 cell proliferation.

Concentration	24 h	48 h	72 h
0 μ M	1.00 \pm 0.040	1.00 \pm 0.038	1.00 \pm 0.039
2 μ M	0.95 \pm 0.034*	0.92 \pm 0.039*	0.89 \pm 0.042*
4 μ M	0.90 \pm 0.035*	0.83 \pm 0.041*	0.78 \pm 0.040*
8 μ M	0.82 \pm 0.036*	0.75 \pm 0.040*	0.62 \pm 0.039*
16 μ M	0.78 \pm 0.040*	0.68 \pm 0.036*	0.57 \pm 0.038*

*P < 0.05 compared to control (0 μ M) group.

Effects of lycopene on MCF-7 cell apoptosis

After treatment of MCF-7 cells with different lycopene concentrations, apoptosis was detected using flow cytometry. Significant difference in cell apoptosis rate was observed between the control and lycopene-treated groups (Figure 2 and Table 2, P < 0.05). Cell apoptosis gradually increased with increasing lycopene concentrations, suggesting a dose-dependent relationship. In addition, longer treatment durations increased the cell apoptotic rate.

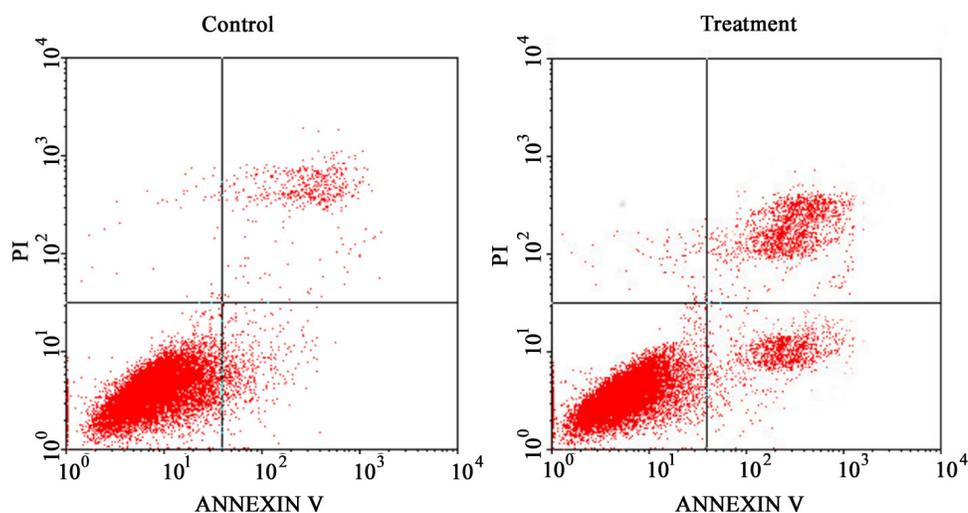


Figure 2. Flow cytometry for cell apoptosis.

Table 2. Effects of lycopene on cell apoptosis.

Concentration	24 h (%)	48 h (%)	72 h (%)
0 μ M	3.12 \pm 1.01	3.26 \pm 1.45	3.45 \pm 1.25
2 μ M	5.26 \pm 1.21*	6.75 \pm 1.34*	6.98 \pm 1.26*
4 μ M	7.35 \pm 1.13*	9.12 \pm 1.47*	11.54 \pm 1.89*
8 μ M	10.12 \pm 2.13*	14.25 \pm 1.78*	18.95 \pm 2.13*
16 μ M	13.14 \pm 1.56*	20.56 \pm 2.15*	28.69 \pm 2.56*

*P < 0.05 compared to control (0 μ M) group.

Effects of lycopene on Bax and p53 mRNA expression levels

The mRNA levels of Bax and p53 in lycopene-treated cells were semi-quantitatively measured using β -actin as the internal reference gene. The mRNA levels of Bax and p53 gradually increased with increasing doses of lycopene and duration of treatment (Tables 3 and 4).

Table 3. Expression of bax mRNA by lycopene.

Concentration	24 h	48 h	72 h
0 μ M	1.00 \pm 0.11	1.00 \pm 0.10	1.00 \pm 0.12
2 μ M	1.13 \pm 0.12	1.24 \pm 0.11*	1.29 \pm 0.10*
4 μ M	1.24 \pm 0.12*	1.28 \pm 0.13*	1.45 \pm 0.12*
8 μ M	1.32 \pm 0.09*	1.42 \pm 0.12*	1.68 \pm 0.13*
16 μ M	1.46 \pm 0.10*	1.60 \pm 0.09*	2.08 \pm 0.14*

*P < 0.05 compared to control (0 μ M) group.

Table 4. Expression of p53 mRNA by lycopene.

Concentration	24 h	48 h	72 h
0 μ M	1.00 \pm 0.09	1.00 \pm 0.08	1.00 \pm 0.10
2 μ M	1.11 \pm 0.13	1.25 \pm 0.12*	1.31 \pm 0.08*
4 μ M	1.26 \pm 0.12*	1.37 \pm 0.13*	1.48 \pm 0.13*
8 μ M	1.43 \pm 0.13*	1.58 \pm 0.14*	1.77 \pm 0.12*
16 μ M	1.50 \pm 0.10*	1.75 \pm 0.11*	1.92 \pm 0.14*

*P < 0.05 compared to control (0 μ M) group.

DISCUSSION

Natural plant extracts have several advantages over chemotherapeutic drugs including fewer adverse effects. In addition, they are suitable for human use. As the anti-tumor components of plant extracts can be obtained through natural diet, their daily dietary intake can help in preventing cancer. Both *in vitro* studies and epidemiology surveys suggested the anti-tumor potency of lycopene (Elgass et al., 2014; Fu et al., 2014; Bhatia et al., 2015; Eliassen et al., 2015; Yan et al., 2016). However, there was inconsistency regarding the exact role of lycopene in breast carcinoma. Therefore, in this study, we aimed to investigate the effect of lycopene in an *in vitro* breast cancer model.

Lycopene treatment resulted in MCF-7 cell shrinkage and damage, which became worse at higher concentrations or longer treatment times or both. MTT assay showed that the activity of MCF-7 cells gradually decreased with increasing lycopene concentrations

and treatment duration. The most significant inhibitory effects were observed in cells treated with 16 μ M lycopene for 72 h. Thus, lycopene showed an inhibitory effect on MCF-7 cell proliferation. Flow cytometry assay showed that lycopene induced the apoptosis of MCF-7 cells in a dose- and time-dependent manner, indicating that lycopene inhibited cell proliferation by inducing MCF-7 cell apoptosis. The results of our study were consistent with those of previous studies, which showed that lycopene can arrest cells at G0/G1 phase to inhibit MCF-7 cell proliferation (Takeshima et al., 2014) and induce apoptosis (Teodoro et al., 2012), and provided further evidences on the anti-tumor effects of lycopene on ER (+) breast cancer.

We also explored the possible mechanisms underlying the effect of lycopene on MCF-7 cells. Our results showed that the mRNA levels of both p53 and Bax increased significantly in a dose- and time-dependent manner after lycopene treatment, suggesting the involvement of p53 and Bax in lycopene-induced MCF-7 cell apoptosis. Previous findings suggested that lycopene could induce the overexpression of Bax in MCF-7 cells (Takeshima et al., 2014). Another study showed decreased expression of Bcl-2 gene, which is closely correlated with Bax function, in MCF-7 cells after lycopene treatment (Li et al., 2002). When overexpressed, the Bcl-2 gene binds to Bax and induces the inhibition of cell apoptosis (Oltvai et al., 1993). These findings supported our conclusion. In addition, increased p53 expression was observed in prostate cancer cells after lycopene treatment (Palozza et al., 2010)

In summary, we demonstrated that lycopene induced apoptosis and inhibited MCF-7 cell proliferation, possibly by regulating the expression of p53 and Bax. However, owing to the inherent complicated mechanisms of breast cancer, future investigations using breast cancer *in vivo* model or other breast cancer cell lines are required to confirm these findings.

Conflicts of interest

The authors declare no conflict of interest.

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